Effects of Fasudil, a Rho-Associated Protein Kinase Inhibitor, on Optic Nerve Head Blood Flow in Rabbits

Tetsuya Sugiyama, Mabo Shibata, Sumiko Kajiura, Takashi Okuno, Masahiro Tonari, Hidehiro Oku, and Tsunehiko Ikeda

PURPOSE. To investigate the effects of fasudil, a Rho-associated coiled coil–forming protein kinase (ROCK) inhibitor, on normal or impaired optic nerve head (ONH) blood flow in a rabbit model.

METHODS. ONH blood flow was measured by laser speckle flowgraphy. Changes in ONH blood flow were examined during a continuous intravenous infusion of fasudil with and without the application of N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor. Effects of topical fasudil on ONH blood flow were investigated in normal eyes or models of ocular circulation impairment induced by the application of endothelin (ET-1). Visual-evoked potentials (VEPs) and morphologic and histologic changes were also analyzed in the ET-1–injected eyes.

RESULTS. A continuous intravenous infusion of fasudil had no significant effect on normal ONH blood flow, yet it prevented or improved the ONH blood flow impairment induced by the intravenous injection of L-NAME. Repeated intravitreal injections of ET-1 twice a week for 4 weeks decreased the ONH blood flow, prolonged the VEPs' implicit time, enlarged the optic cup, and decreased retinal ganglion cells. Multiple doses of topical fasudil ameliorated the ONH impairments caused by ET-1.

CONCLUSIONS. These results show that systemic or topical fasudil suppresses impairment of ONH blood flow, function, and morphology induced by L-NAME or ET-1. A ROCK inhibitor can be useful for the treatment of impaired ONH blood flow.

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Regulation of basal vascular tone is dependent on the balance of vasoconstriction and vasodilation. Contraction of smooth muscle cells is initiated by the phosphorylation of the myosin light chain (MLC), which then allows the interaction of myosin and smooth muscle actin. Phosphorylation of the MLC is dependent on the activation of the MLC kinase and the MLC phosphatase. The MLC kinase is a calcium/calmodulin-activated enzyme, whereas the MLC phosphatase is calcium independent and inhibited by phosphorylation through the Rho-associated coiled coil–forming protein kinase (ROCK). Cell culture experiments and studies using isolated vessel preparations demonstrated that the constrictor effects of endothelin and angiotensin II and the generation of myogenic tone are mainly mediated by ROCK activation.

The critical role of ROCK in the control of smooth muscle constrictor tone, however, may suggest that this enzyme also has a major function in the control of vascular tone under physiological conditions. Besides its profound effects on vascular smooth muscle cells, ROCK is also involved in the regulation of endothelial nitric oxide (NO) synthase (eNOS). In human endothelial cells, ROCK attenuates phosphorylation of eNOS through inhibition of protein kinase B/Akt. Moreover, inhibition of ROCK leads to phosphorylation and an activation of Akt via the phosphatidylinositol 3-kinase, ultimately resulting in an increased production of NO. These data suggest that ROCK plays an important role in the regulation of eNOS in the peripheral circulation.

Although several reports indicated the intraocular pressure (IOP)-lowering effects of ROCK inhibitors, to the best of our knowledge their effects on ocular blood flow have yet to be reported. Fasudil, a selective ROCK inhibitor, is clinically used in several countries for the treatment of cerebral vasospasms after subarachnoid hemorrhage. Several studies have demonstrated that fasudil increases cerebral blood flow and has a beneficial effect for the treatment of acute and/or chronic cerebral ischemic stroke and vasospasm. Systemic administration of fasudil was reported to have vasodilator effects on retinal arterioles in stroke-prone spontaneously hypertensive rats. It is also reported that fasudil protects the vascular endothelium by inhibiting neutrophil adhesion and reducing neutrophil-induced endothelial injury in diabetic rats.

The aim of this present study was to test whether systemic or topical application of fasudil has effects on basal or disturbed optic nerve head (ONH) circulation in a rabbit model.

MATERIALS AND METHODS

Animals

Male albino rabbits weighing 2.5 to 3.4 kg were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). They were housed in an air-conditioned room at 22 ± 1°C and 66 ± 5% humidity with a 24-hour light–dark cycle. They were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Chemicals

Unless otherwise noted, the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Fasudil was supplied by Asahi Kasei Pharma Co. (Tokyo, Japan). Human endothelin (ET)-1 was purchased from the Peptide Institute (Osaka, Japan). As supplied, each vial contained 0.11 mg of ET-1, which was dissolved in 0.44 mL of 0.1% aqueous acetic acid to provide a 10⁻⁷ M solution. The ET-1 concentration was adjusted to 10⁻⁷ M by further dilution with a balanced saline solution.
Experimental Protocol

For the first experiment, the effects of continuous intravenous infusions of fasudil (0.01, 0.05, or 0.1 mg/kg/min) or physiological saline into an auricular vein for 30 minutes on the ONH blood flow, IOP, and systemic blood pressure were examined in six eyes of six rabbits for each dose of fasudil or physiological saline. A syringe pump (Terufusion syringe pump, TE-311; Terumo, Tokyo, Japan) was used for the continuous infusion. The doses of fasudil used in this study were decided based on the fact that fasudil is clinically used at 1.0 mg/person/min. The rabbits were placed in holding boxes, and the measurements were obtained every 10 minutes for 1 hour under local anesthesia with a drop of 0.4% oxybuprocaine hydrochloride (Benoxil; Santen Pharmaceutical Co., Osaka, Japan).

For the second experiment, a continuous intravenous infusion of fasudil (0.03 mg/kg/min) was started just before or 30 minutes after an intravenous injection of 1 mL N^6-nitro-L-arginine methyl ester (L-NAME) (3 or 10 mg/kg), a nitric oxide synthase inhibitor. The dose of L-NAME was determined based on the results of our previous report.20 The ONH blood flow was measured just before and after the injection of L-NAME every 10 minutes for 1 hour under local anesthesia as described above. In addition, it was examined whether wortmannin (0.6 mg/kg), a specific inhibitor of Akt/ phosphatidylinositol (PI)-3 kinase, simultaneously administered with L-NAME (10 mg/kg), inhibits the effect of fasudil on the ONH blood flow. In six rabbits infusion of fasudil was initiated just before the injection of L-NAME (5 mg/kg), and another six rabbits received only the L-NAME (3 mg/kg). Four rabbits received fasudil 30 minutes after the L-NAME (10 mg/kg) injection, whereas another four rabbits received wortmannin simultaneously with L-NAME (10 mg/kg).

For the third experiment, the effects of multiple administrations of fasudil eye drops (10% solution dissolved in physiological saline, 50 μL) twice daily (in the morning and in the evening) for 8 weeks on the ONH blood flow, IOP, visually evoked potentials (VEPs), ONH morphology, and densities of retinal ganglion cells (RGCs) were examined in normal eyes (n = 4) or the eyes with impaired ONH blood flow (n = 7). To determine the concentration of fasudil to be used as an eye drop, we performed the preliminary experiment comparing the effects of 5% and 10% fasudil; the mean ± SE values of the means (SEM) of the relative NB values compared with the pretreatment level were 113.7 ± 9.2 and 122.6 ± 10.0 at 4 hours after the instillation of 5% and 10% fasudil in 3 rabbits. The increment induced by 10% fasudil was larger than that by 5% fasudil though the difference was not significant (P > 0.05; unpaired t-test). Accordingly, we decided to use 10% fasudil because this concentration seems to be nearly the maximum that can be dissolved for the agent. The fellow eye of each rabbit was given the vehicle for fasudil (physiological saline). The impairment of ONH blood flow was induced by intravitreal injection of ET-1 (10^{-6} M, 20 μL, 20 pmol) twice a week (Tuesday and Friday) for 4 weeks. Measurements of blood flow, visual function, IOP, and systemic blood pressure were performed every Tuesday (before the ET-1 injection on that day) and also approximately 1 to 3 hours after the instillation of fasudil or its vehicle in the morning. Those measurements were also performed in sham control eyes (n = 4), which were given only intravitreal injection of the vehicle for ET-1, instead of ET-1 itself. These time courses and procedures were adjusted to those in our previous report.21 The same investigator (TS) carried out these procedures throughout the study. This investigator was blinded to the instillation given to the various rabbits.

Measurement of ONH Blood Flow

ONH blood flow was measured with the recently developed laser speckle flowgraphy, which permits noninvasive and two-dimensional measurements of tissue circulation in the ONH, choroid–retina. The laser speckle flowgraphy apparatus consists of a fundus camera equipped with a diode laser (wavelength 808 nm) and an image sensor (100 × 100 pixels). The principle and application of this method has been described previously.22-25 The normalized blur (NB) value in the ONH free of visible surface vessels indicates the blurring of the speckle pattern formed by scattered light, which was originally considered to represent a quantitative index of blood velocity in the microcirculation. Alternations in the NB values are thought to represent changes in capillary blood flow in the ONH, because the NB values have been shown to correlate well with changes in the ONH blood flow assessed by the hydrogen gas clearance method.24 In the present study, the NB values were recorded five times at each time point, and then the mean value was calculated. The ONH blood flow was measured after dilating the pupil with one drop of 0.5% tropicamide (Mydrin M; Santen). During the measurement, the eye to be measured was held open with a Barraquer wire speculum under local anesthesia with oxybuprocaine hydrochloride (Benoxil; Santen).

Measurement of IOP and Mean Blood Pressure

IOP was measured with a calibrated pneumotonometer (Model 30 Classic; Medtronic ENT Ophthamalics, Jacksonville, FL) under local anesthesia with oxybuprocaine hydrochloride. Mean blood pressure (MBP) was measured at the front leg by an automatic sphygmomanometer (BP-98E; Softron, Tokyo, Japan). A close correspondence between the pressure determined by this sphygmomanometer and that obtained through a pressure transducer cannula placed in the femoral artery was confirmed previously.25 Ocular perfusion pressure (OPP) was determined as MBP minus IOP.

Recording of VEPs

To assess the changes of optic nerve function, VEPs were recorded. The method used for recording VEPs has been described in detail elsewhere.26 Briefly, VEPs were elicited by 0.6 J photic stimuli using a photic stimulator (SLS 4100; Nikon-Kohden, Tokyo, Japan) in conscious rabbits placed in holding boxes. The signals were amplified with the bandpass filters of a biophysical amplifier (AVM-10; Nikon-Kohden) set at 1.5 to 100 Hz, and a signal averager (DAT-1100; Nikon-Kohden) was used to summate 32 responses. The pupil of the eye to be recorded was fully dilated with 0.4% tropicamide, and the eye was held open with a Barraquer wire speculum. A diffuser was placed before the stimulated eye to ensure full-field stimulation, and the mean luminance at the corneal surface was 0.42 lux-second. The active stainless-steel electrode was placed on the dura at 6 mm anterior and 6 mm left or right of the lambda point (for a right or left eye, respectively), with the reference stainless-steel electrode placed on the midline 16 mm anterior to lambda. Implantation of an active and reference electrode was performed under general anesthesia (intraperitoneal urethane, 0.8 g/kg) at least 2 weeks before the experiment. Responses were elicited by stimulating only one of the eyes and recorded from the active electrode, while the fellow eye was completely obstructed to prevent stray-light stimulation. Rabbits were grounded by an electrode attached to one of the ears.

Analog data were recorded on a rectilinear pen recorder and also fed in parallel to a microcomputer system to be digitized and stored for later analysis (MacLab 2e; AD Instruments, Castle Hill, Australia). The implicit time (IT) and amplitude of the first negative peak (N1), which was the most prominent peak and appeared approximately 20 ms after stimulation, were automatically calculated by the computer program using the stored data. This analysis was done by one of the authors (TO) in a masked fashion.

Morphologic Analysis

The morphology of the ONH was analyzed using fundus pictures taken from different angles before and 8 weeks after the first ET-1 injection, using a method described elsewhere.27 Briefly, color fundus photographs were taken (1) with the ONH at the center and (2) with the upper margin of the ONH at the center of the field using a fundus camera at a 45° visual angle. The photographs were digitized, fed into a microcomputer, and rotated 90° clockwise. A computer program then configured the images to provide a stereoscopic image of the ONH. While viewing a stereographe, the optic disc area (DA) and the
optic cup area (CA) were drawn by circles as precisely as possible. These areas were then measured by calculating the number of pixels in each area on the computer display using National Institutes of Health image software, and the change in the CA/DA ratio was taken as an index of cup enlargement. The stereographs were analyzed randomly in a masked fashion by one of the authors (HO).

### Histologic Analysis

After completion of the 8-week experiment of an ET-1 injection model, rabbits were killed by intravenous injection of a lethal dose of pentobarbital sodium. The eyes were then enucleated, fixed in 2% paraformaldehyde-2.5% glutaraldehyde in 10 mM PBS, rinsed with 10 mM PBS, and embedded in paraffin. Transverse sections of the retina (180 m) were cut parallel to the medullary rays, 2 mm directly inferior to the center of the ONH, and stained with hematoxylin and eosin. To evaluate the damage to RGCs, RGCs around the center of the retina were counted by a masked colleague not associated with the experiment. For the analysis, 10 light photomicrographs taken at 180x260 magnification around the center of each retinal slice over a distance of approximately 5 mm were provided in a masked fashion. The examiner (SK) then counted all the RGCs in these pictures in a masked fashion.

### Statistical Analyses

The data are expressed as mean ± SEM unless otherwise noted. Statistical analysis was performed using one- or two-way ANOVA, paired or unpaired t-test. Differences were accepted as significant for $P < 0.05$.

### RESULTS

#### Effects of Intravenous Infusions of Fasudil on Normal Eyes

There was no significant difference between the ONH blood flow changes induced by continuous intravenous infusions of fasudil (0.01, 0.03, or 0.1 mg/kg/min) or physiological saline for 1 hour. The middle dose of fasudil (0.03 mg/kg/min) tended to increase the blood flow, but not significantly (Fig. 1). MBP, IOP, and OPP were significantly reduced by continuous intravenous infusions of fasudil at 0.1 mg/kg/min compared with the initial level (Table 1). Other doses of fasudil tended to reduce MBP and IOP, but not significantly (data not shown).

#### Effects of Intravenous Infusions of Fasudil on Impaired Ocular Circulation

A continuous intravenous infusion of fasudil (0.03 mg/kg/min) prevented the impairment of ONH blood flow for at least 1 hour when it was started just before an intravenous injection of 1 mL of L-NAME (3 mg/kg) (Fig. 2A). It ameliorated the impairment significantly when the infusion was started 30 minutes after the injection of L-NAME (10 mg/kg), whereas wortmannin inhibited the amelioration. (Fig. 2B). The mean ± SEM of MBP for 4 rabbits was 110.0 ± 6.8, 104.0 ± 12.1, 107.6 ± 12.2, 108.0 ± 11.1 (before and at 10, 20, and 30 minutes after the injection of 10 mg/kg of L-NAME, respectively), which showed no significant change.

#### Effects of Multiple Doses of Topical Fasudil on Normal or Chronically Impaired Ocular Circulation

The multiple administration of topical fasudil did not have any significant effects on the ONH blood flow of normal eyes when measured at 1 to 3 hours after instillation (ANOVA, $P < 0.05$, data not shown), yet OPP was not significantly affected (Fig. 3B). It likewise did not significantly change IT of N1 and the density of RGCs in the normal eyes (ANOVA, $P < 0.05$), control). Data are expressed as mean ± SEM for six rabbits. IOP, intraocular pressure; MBP, mean blood pressure; OPP, ocular perfusion pressure.

### Table 1. Changes in MBP, IOP, and OPP after Intravenous Injection of Fasudil

<table>
<thead>
<tr>
<th>Fasudil (0.1 mg/kg/min)</th>
<th>0 min</th>
<th>20 min</th>
<th>40 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP (mmHg)</td>
<td>92.3 ± 3.4</td>
<td>81.0 ± 3.8*</td>
<td>88.6 ± 5.4</td>
<td>89.5 ± 3.9</td>
</tr>
<tr>
<td>IOP (mmHg)</td>
<td>19.1 ± 0.8</td>
<td>17.9 ± 0.8</td>
<td>15.7 ± 0.9*</td>
<td>17.3 ± 1.2</td>
</tr>
<tr>
<td>OPP (mmHg)</td>
<td>73.2 ± 5.2</td>
<td>63.1 ± 3.6*</td>
<td>72.9 ± 5.0</td>
<td>72.2 ± 3.8</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM for six rabbits. IOP, intraocular pressure; MBP, mean blood pressure; OPP, ocular perfusion pressure.
* Significant changes ($P < 0.05$, Dunnett’s test) compared with the initial value (0 min).
control eyes remained stable throughout the study (Fig. 4A). IOP was almost stable in the sham control and ET-1/vehicle-treated eyes, whereas it was reduced in the ET-1/fasudil–treated eyes. There were significant differences in IOP between the ET-1/fasudil–treated eyes and the other two groups (ANOVA, \( P < 0.05 \), data not shown). Changes in OPP are shown in Figure 4B. There were no significant differences in OPP between the three groups (ANOVA, \( P > 0.05 \)). IT of N1 was stable in the sham control eyes. On the other hand, it gradually prolonged in the ET-1/vehicle–treated eyes, whereas the prolongation was prevented in the ET-1/fasudil–treated eyes (ANOVA, \( P < 0.05 \); Fig. 4C). The changes in ONH morphology are shown in Table 2. In the ET-1/vehicle-treated eyes, CA/DA was significantly increased, but such changes were not observed in the ET-1/fasudil–treated eyes. The density of RGCs in the retinas of the ET-1/vehicle–treated eyes was significantly decreased (paired \( t \)-test, \( P < 0.01 \)) compared with the sham control and ET-1/fasudil–treated eyes (Fig. 4D).

**DISCUSSION**

This present study revealed that a continuous intravenous infusion of fasudil had no significant effect on normal ONH blood flow, although it prevented or improved the impairment of ONH blood flow induced by an intravenous injection of L-NAME. The results of this study also showed that a single or repeated application of topical fasudil prevented the impairment of ONH blood flow induced by L-NAME or ET-1, and that repeated application of topical fasudil also inhibited prolongation in the VEP implicit time, enlargement in the optic cup, and reduction in the number of RGCs.

The methods used in this study (laser speckle flowgraphy and VEP) have already been shown to obtain valid data in rabbits. Laser speckle flowgraphy was developed in Japan for assessing the blood flow in the ONH, choroid, and retina, and its accuracy has been demonstrated in rabbits.22,23 In addition, a previous report has demonstrated the accuracy of IT and N1 in rabbit VEPs.26

In this present study, no significant effect of systemically applied fasudil on the normal ONH blood flow was detected, though a certain dose tended to increase the blood flow. Fasudil has been reported to dilate various kinds of vessels, thus reducing systemic blood pressure; because it subsequently also decreased OPP, the increase in normal ONH blood flow might possibly also be prevented.
We intravenously injected L-NAME, an NOS inhibitor, to induce impairment in ONH blood flow as shown in the previous report. Intravenous fasudil not only prevented the impairment of ONH blood flow when L-NAME was injected just after the start of treatment with fasudil, but also ameliorated its impairment induced by intravenous L-NAME injected 30 minutes before the treatment with fasudil in the present study. Because the amelioration was inhibited by a specific inhibitor of Akt/PI-3 kinase, we theorize that these actions of fasudil might be caused by inhibition of ROCK, leading to phosphorylation and an activation of Akt via the PI-3 kinase. A possible explanation why intravenous infusion of fasudil had a vasodilatory effect on ONH blood flow under the condition of NOS inhibition though it did not change ONH blood flow significantly under the basal condition might be one of the following: (1) Systemic blood pressure tended to increase after NOS inhibition, and therefore it was not reduced by fasudil compared with the previous level, differently from the case of basal condition, as discussed above. (2) Rho-kinase is reportedly activated in vascular cells in a rat cerebral infarction model, and therefore the inhibitory effect of ROCK against Rho-kinase might be more powerful in the condition of ONH blood flow impairment after NOS inhibition. Topical fasudil also prevented the ONH blood flow impairment induced by ET-1 injection. Again we can explain the reason for the discrepancy between the effects of topical fasudil on normal eyes and ET-1-treated eyes by the increased Rho-kinase activity in vascular cells of impaired ONH blood flow, as mentioned above. Although there is currently no data on the local penetration of instilled fasudil solution into the posterior segment of the eye, the maximum concentrations of fasudil in the cornea and aqueous humor after a single instillation of 1% solution were reported to be approximately 283 and 20 μM, respectively (Hisashi Aratake 2005, personal communication). These data suggest a higher intraocular penetration of fasudil than that of timolol (the maximum concentrations in the cornea and aqueous humor after instillation of 1% timolol were reportedly 8.85 and 0.63 μM, respectively) but does not warrant a high penetration of fasudil into the posterior segment of the eye. This matter requires further investigation. As another limitation of the present study, our results did not exclude the possibility that the amelioration of ONH blood flow was independent from the direct effect of fasudil on the microcirculation in ONH because we performed only in vivo studies.

The findings of this study also revealed that multiple doses of topical fasudil suppressed the impairment in ONH blood flow as well as the prolongation in IT of N1, the morphologic change in ONH, and the decrease in the density of RGCs. These results suggest that fasudil produces not only a preferable effect on ONH blood flow but also a neuroprotective effect. Although the direct inhibition of ROCK in vascular smooth muscle by fasudil could contribute to the acute or chronic vasodilator response, it is unknown whether the vasodilative action might be involved in the neuroprotective effects of fasudil. This issue warrants further investigation in a future study.

Topically applied fasudil reduced IOP in the present study, similar to the findings previously reported. According to that previous report, the IOP-lowering effects of fasudil may be related to the altered cellular behavior of trabecular meshwork cells and the relaxation of ciliary muscle contraction. As another ROCK inhibitor, SNJ-1656, is reportedly now under development as an IOP-lowering drug, we theorize that fasudil might also prove to be a candidate drug for treating glaucoma. The findings in this present study suggest that fasudil might be an alternative treatment for glaucoma.

**TABLE 2.** Changes in the Optic Cup Area/Optic Disc Area Ratio

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>8 Weeks after Start of ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>0.27 ± 0.03</td>
<td>0.34 ± 0.05*</td>
</tr>
<tr>
<td>ET-1 + fasudil</td>
<td>0.29 ± 0.05</td>
<td>0.28 ± 0.04</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM for seven rabbits.
* Significant difference from baseline value (P < 0.05, paired t-test).

**FIGURE 4.** Changes in NB values of the ONH (A), OPP (B), and implicit time of visually evoked potentials (C) after intravitreal injection of ET-1 (20 pmol) with multiple administrations of 50 μL of 10% fasudil (D) or physiological saline (C), or after intravitreal injection of the vehicle (20 μL) for ET-1 (E). Data are expressed as mean ± SEM for four to seven rabbits. The two-way interaction between the ET-1 + vehicle-treated and the ET-1 + fasudil-treated groups was significant for (A) and (C) (P < 0.01 and P < 0.05, respectively). The two-way interaction between the sham control and the ET-1 + fasudil-treated groups was significant for (A) by ANOVA (P < 0.05), but not for (B) or (C). Asterisks indicate significant differences between the two bracketed groups (paired t-test, *P < 0.05, **P < 0.01).
effective for ocular diseases in which impairment in ONH blood flow is involved, such as glaucoma or ischemic optic neuropathy, although further studies are needed to verify its clinical usefulness.

In summary, our results suggest that a systemic or topical application of the ROCK inhibitor fasudil may suppress the impairment of ONH circulation, as well as that of optic nerve function and morphology, and may be effective for the treatment of ocular diseases with impaired ONH blood flow, including glaucoma.

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References


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